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Human PTH-(7-84) Inhibits Bone Resorption *in Vitro* Via Actions Independent of the Type 1 PTH/PTHrP Receptor

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The linear sequence of intact mammalian PTH consists of 84 amino acids, of which only the most amino(N)-terminal portion, *i.e.* PTH-(1-34), is required for the classical actions of the hormone on mineral ion homeostasis mediated by the type 1 PTH/PTHrP receptor (PTH1R). Like the N-terminus, the carboxyl (C)-terminal sequence of PTH is highly conserved among species, and various circulating PTH C-fragments are generated by peripheral metabolism of intact PTH or are directly secreted, in a calcium-dependent manner, by the parathyroid glands. Certain synthetic PTH C-fragments exert actions on bone and cartilage cells that are not shared by PTH-(1-34), and specific binding of PTH C-peptides has been demonstrated in bone cells in which PTH1R expression was eliminated by gene targeting. The peptide human (h) PTH-(7-84) recently was shown to inhibit the calcemic actions of hPTH-(1-34) or hPTH-(1-84) in parathyroidectomized animals. To determine whether this anticalcemic effect of hPTH-(7-84) *in vivo* might result from direct actions on bone, we studied its effects on both resorption of intact bone *in vitro* and formation of osteoclasts in primary cultures of murine bone marrow. Human (h) PTH-(7-84) (300 nM) reduced basal 72-h release of preincorporated ^{45}Ca from neonatal mouse calvariae by 50% (9.6 ± 1.9% *vs.* 17.8 ± 5.7%; $P < 0.001$) and similarly inhibited resorption induced by hPTH-(1-84), hPTH-(1-34), 1,25-dihydroxyvitamin D_3 (VitD), PGE_2, or IL-11. In 12-d murine marrow cultures, both hPTH-(7-84) (300 nM) and hPTH-(39-84) (3000 nM) lowered VitD-dependent formation of osteoclast-like cells by 70%. On the contrary, these actions of hPTH-(7-84) were not observed with the PTH1R antagonists hPTH-(3-34)NH_2 and [L^{11},D-W^{12},W^{23},Y^{36}]hPTHrP-(7-36)NH_2, which, unlike hPTH-(7-84), did inhibit PTH1R-dependent cAMP accumulation in ROS 17/2.8 cells. We conclude that hPTH-(7-84), acting via receptors distinct from the PTH1R and presumably specific for PTH C-fragments, exerts a direct antiresorptive effect on bone that may be partly due to impaired osteoclast differentiation. (*Endocrinology* 143: 171–176, 2002)

INTACT PTH from different mammalian species comprises 84 amino acids, the sequence of which is highly conserved within both its amino (N)-terminal and carboxyl (C)-terminal regions (1). The first 34 amino acids of PTH, including an intact N-terminus, are both necessary and sufficient for the classical actions of the hormone on mineral ion homeostasis and bone metabolism. These effects of intact and N-terminal PTH are mediated through the type 1 PTH/PTH-related peptide receptor (PTH1R), a G protein-coupled receptor that can activate both adenylate cyclase and PLC (2).

The parathyroid glands are the main source of PTH, although small amounts of its mRNA were recently identified in hypothalamus and spleen (3). PTH synthesis and secretion are tightly controlled by calcium via a membrane-bound calcium-sensing receptor (4), although vitamin D (5, 6) and phosphate (7, 8) also play modulating roles. Under physiological conditions, a portion of the newly synthesized hormone undergoes intraglandular cleavage at a rate that also is regulated by extracellular calcium (9, 10). This cleavage results in the cosecretion of intact PTH and various C-terminal fragments, the predominant forms of which, identified to date, consist of peptides with N-termini located between residues 24 and 43 (11–13). Secreted intact PTH also undergoes endopeptidic cleavage(s) in peripheral tissues, mainly liver and kidney, by processes that degrade the resulting N-terminal fragments *in situ* but release additional C-fragments into the circulation (14–16). As a consequence of their obligatory renal clearance, the concentration of circulating C-terminal PTH (CPTH) fragments increases dramatically in patients with renal failure (17–19).

Recently, fragments of PTH lacking residues at the extreme N-terminus but otherwise large enough to cross-react with most commercially available intact PTH two-site immunoassays were detected after HPLC fractionation of normal plasma and, at much higher levels, in plasma of patients with advanced renal failure (19). Although their precise structure(s) has not been ascertained, these fragments exhibit chromatographic properties similar to those of synthetic PTH-(7-84) (18). Interestingly, human (h) PTH-(7-84) was recently shown to inhibit the calcemic actions of PTH-(1-84) and PTH-(1-34) in parathyroidectomized animals at doses much lower than would be predicted to effectively antagonize either hormonal form at the PTH1R (20, 21). Thus, these *in vivo* observations suggest that CPTH fragments might act upon bone cells via one or more mechanisms independent of the PTH1R *per se*.

The possibility that CPTH fragments (as well as intact PTH) might activate receptors distinct from the PTH1R was first postulated over 2 decades ago when Arber *et al.* (22) showed that a particular CPTH fragment, PTH-(53-84), possessed biological properties different from those of PTH-(1–34). Subsequent work from several different groups has produced direct evidence that CPTH fragments from within the sequence PTH-(35-84) bind specifically to bone and kidney cells and/or membranes and can exert direct actions on target cells in bone or cartilage. For example, CPTH frag-

Abbreviations: CPTH, C-terminal PTH; hPTH, human PTH; PTH1R, type 1 PTH/PTHrP receptor; TRAP, tartrate-resistant acid phosphatase; TRAP+MNC, TRAP-positive cells containing three or more nuclei (osteoclast-like multinucleated cells); VitD, 1,25-dihydroxyvitamin D_3.

ments such as hPTH-(53-84) and hPTH-(60-84) increased alkaline phosphatase activity and expression of mRNAs for both alkaline phosphatase and osteocalcin in bone-derived cells and induced transient increases in cytosolic free calcium in chondrocytes (23–26). Photoaffinity cross-linking studies to characterize the receptors for CPTH fragments (*i.e.* CPTHRs) expressed by ROS 17/2.8 osteosarcoma and rPT parathyroid cells were performed by Inomata *et al.* (27) using radioiodinated (Leu^{8,18},Tyr^{34})hPTH-(1-84) and (Tyr^{34})hPTH-(19-84), neither of which binds well, if at all, to the PTH1R. These studies showed that in ROS 17/2.8 cells, two proteins (80 and 30 kDa) interacted specifically with the radioligands used, whereas in rPT cells, only the 80-kDa protein was observed. Affinity labeling was inhibited by hPTH-(1-84), hPTH-(19-84), and, to a lesser extent, by CPTH fragments that were truncated even further at the N-terminus, whereas hPTH-(1-34) had no effect (27). Recently, hPTH-(7-84) was shown to bind to CPTHRs on ROS 17/2.8 cells with affinity comparable to that of hPTH-(1-84) (21).

Unequivocal evidence that such CPTHRs are distinct from the PTH1R was provided by our recent demonstration that specific [^{125}I](Tyr^{34})hPTH-(19-84) binding is observed in clonal osteoblastic and osteocytic cell lines derived from mice in which the PTH1R gene had been eliminated by gene targeting (28). Further, CPTH fragments such as hPTH-(39-84) were shown to regulate cellular functions (*i.e.* connexin 43 expression and apoptosis) in clonal PTH1R-null osteocytes at concentrations shown to bind effectively to CPTHRs in these cells (28).

Thus, the expression of CPTHRs in bone offers a plausible mechanism by which circulating PTH fragments, truncated at their N-termini and including peptides as long as hPTH-(7-84) might exert biological actions, potentially different from those of intact PTH, by a means other than direct antagonism at the PTH1R. To determine whether the ability of hPTH-(7-84) to antagonize the calcemic response to PTH-(1-84) *in vivo* might reflect direct actions of this C-PTH fragment on bone, we studied its effects using *in vitro* assays of osteoclast formation and bone resorption.

Materials and Methods

Materials

Culture media were obtained from the Media Kitchen (Pediatric Surgery, Massachusetts General Hospital, Boston, MA), other tissue culture reagents were purchased from Life Technologies, Inc. (Grand Island, NY), and additional reagents and chemicals were obtained from Sigma (St. Louis, MO) or Fisher (Pittsburgh, PA). Recombinant hPTH-(1-84) was a gift from Chugai Pharmaceutical Co. (Shizuoka, Japan), and hPTH-(7-84) and [D^{76}]hPTH-(39-84) were purchased from Bachem (Torrance, CA). All other PTH fragments, including the PTH1R antagonist (Leu^{11},D-Trp^{12},Trp^{23},Tyr^{36})hPTHrP-(7-36)amide PTHrP-(7-36) (29, 30) and hPTH-(3-34)-amide, were synthesized at Massachusetts General Hospital Peptide and Oligonucleotide Core Laboratory (Boston, MA). Recombinant mouse IL-11 was purchased from R&D Systems, Inc. (Minneapolis, MN), and VitD was obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA).

Animals

Animals were maintained in facilities operated by the Massachusetts General Hospital Center for Comparative Resources in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were employed using protocols approved by the institutional animal care and use committee.

Bone resorption assay

Bone resorption was quantitated by the release of previously incorporated ^{45}Ca from newborn mouse calvarial bones *in vitro* (31). Briefly, calvaria from 3- to 4-d-old mice (CD-1 strain, Charles River Laboratories, Inc., Wilmington, MA) were obtained after maternal administration of 50 μCi $^{45}CaCl_2$ (NEN Life Science Products, Boston, MA), sc, on the 19th day of gestation. The bones were divided in half and precultured in 1 ml DMEM containing 1 mM calcium, 2 mM phosphate, 5% heat-inactivated horse serum, and 1% antibiotic/antimycotic solution (Life Technologies, Inc.) on a rocking platform at 90 oscillations/min in a 37 C incubator under 5% CO_2 in air. After 24 h the medium was replaced with 1 ml fresh medium containing the test substances (or vehicle alone). After an additional 72 h, the bones were removed, rinsed three times in PBS, placed in scintillation vials containing 0.4 ml 2 N HCl, and incubated for 2 h at room temperature before addition of 5 ml scintillation fluid (Packard Instruments, Downers Grove, IL). Aliquots of culture medium (0.5 ml) were transferred to separate vials containing 5 ml scintillation fluid for determination of released radioactivity. In some experiments additional aliquots of culture medium were used for measurements of cAMP as described below. Bone resorption was determined as the percentage of total initial bone ^{45}Ca subsequently released into the medium during the 72-h treatment period. Results are expressed as the mean ± SEM of the percentage of ^{45}Ca released for groups of four bones and are representative of at least three independent experiments.

Bone marrow culture

Bone marrow cells were isolated as previously described (32). Briefly, 4- to 6-wk-old male mice (C57B/6 strain, Charles River Laboratories, Inc.) were killed by carbon dioxide asphyxiation, and tibias and femurs were aseptically removed and dissected free of adhering tissue. The metaphyses were removed, and the marrow cavity was flushed with 1 ml αMEM to obtain marrow cells, which were collected into 50-ml tubes and washed twice with αMEM. Cells were cultured in growth medium [αMEM containing 10% FBS (lot 1011961 Life Technologies, Inc.) and 1% penicillin-streptomycin] containing 100 nM dexamethasone (Sigma) after plating at 1.5×10^6 cells/well in 24-well plates. Half of the culture medium was replaced 3 times/wk with fresh medium containing a 2× concentration of the test substances (or vehicle). All cultures were maintained in a 37 C incubator under 5% CO_2 in air. After culture for 10 d, cells adherent to the surface of each well were rinsed twice with PBS, fixed with 10% formalin in PBS for 10 min at room temperature and with ethanol/acetone (50:50, vol/vol) for 1 min before staining for tartrate-resistant acid phosphatase (TRAP), as previously described (33). TRAP-positive cells containing 3 or more nuclei were scored as osteoclast-like multinucleated cells (TRAP+MNCs). Cells were counted at ×10 magnification in 20 contiguous fields along 2 orthogonal pathways in each well, a method previously employed to account for the nonuniform distribution of cells within wells (33). The number of TRAP+MNCs contained in these 20 fields was expressed as the number per well.

cAMP accumulation

Clonal rat osteosarcoma cell (ROS 17/2.8) were cultured in 48-well plates in Ham's F-12 medium (Life Technologies, Inc.) supplemented with 10% FBS and 1% penicillin-streptomycin. The cultures were maintained for 5–7 d after reaching confluence by replacing the medium every other day. To assess basal and agonist-induced cAMP accumulation, cells were rinsed twice with assay buffer (DMEM containing 2 mM isobutylmethylxanthine, 1 mg/ml heat-inactivated BSA, and 35 mM HEPES-NaOH, pH 7.4) and then incubated for 45 min at 23 C with the same buffer alone or in the presence of different peptides (or with conditioned medium collected from resorption assays). The buffer then was rapidly aspirated, the plates were frozen on powdered dry ice, and the frozen cells were subsequently thawed directly into 0.25 ml 50 mM HCl. Cell-associated cAMP in the acid extracts was measured as previously described (34). Results were expressed as picomoles of cAMP produced per well over 45 min.

Statistical analysis

Results are expressed as the mean ± SEM or the mean ± SD. The significance of differences between treatment and control groups was

assessed by the Mann-Whitney test. Data were analyzed using the PRISM 3.0 software package for Macintosh (GraphPad Software, Inc., San Diego, CA).

Results

The limited efficacy of short, amino-terminally truncated PTH or PTHrP analogs, such as hPTH-(3-34) or hPTHrP-(7-36), to antagonize the action of PTH-(7-34) or PTH-(1-84) *in vivo* or *in vitro* contrasts with the efficiency with which hPTH-(7-84) inhibits the calcemic action of hPTH-(1-84) [or hPTH-(7-34)] *in vivo* (20, 21). Although pharmacokinetic differences *in vivo* between N-truncated PTH fragments of different length and structure might explain these differences, we considered the alternative possibility that, unlike short N-truncated PTH or PTHrP fragments, this effect of hPTH-(7-84) *in vivo* might not be mediated via antagonism at the PTH1R. We therefore directly compared the effects of hPTH-(7-84) with those of PTHrP-(7-36) or hPTH-(3-34) in an *in vitro* assay of bone resorption that relies upon the release of pre-incorporated ^{45}Ca from neonatal murine calvarial bones. First, as shown in Fig. 1, addition of hPTH-(7-84) (300 nM) alone reduced basal ^{45}Ca release by approximately 50% [control, 17.8 ± 5.7%; hPTH-(7-84), 9.6 ± 1.9%; $P < 0.001$]. This effect was comparable to that of salmon calcitonin (100 nM; 9.9 ± 1.1%; $P < 0.001$). In contrast, no inhibition of basal resorption was observed with equimolar concentrations of much shorter N-truncated PTH analogs, such as hPTH-(3–34) (300 nM; 18.7 ± 4.2%) or PTHrP-(7-36) (300 nM; 15.4 ± 4.9%), that bind as well or more effectively to the PTH1R as hPTH-(7-84).

Further, as shown in Fig. 2, hPTH-(7-84) (300 nM) also significantly inhibited (by 50% or more) agonist-induced bone resorption caused by a variety of osteotropic agents, including intact hPTH-(1-84) (3 nM), hPTH-(1-34) (3 nM), VitD (10 nM), PGE_2 (100 nM), and IL-11 (10 ng/ml). The antiresorptive effect of hPTH-(7-84) was dose dependent, with an IC_{50} of approximately 200 nM (data not shown). We considered the possibility that these inhibitory actions of hPTH-(7-84) might reflect antagonism, at the PTH1R, of an effect of locally secreted PTHrP to augment the responses to these other agonists. As shown in Fig. 3, however, neither hPTH-(3-34) (1 μM) nor PTHrP-(7-36) (1 μM), both of which act as PTH1R antagonists (see below) at the concentrations used, inhibited resorption induced by VitD (10 nM). With respect to a possible nonspecific or irreversible toxic effect of hPTH-(7-84), we observed that removal of the peptide after 24 h of exposure to calvariae did not impair the

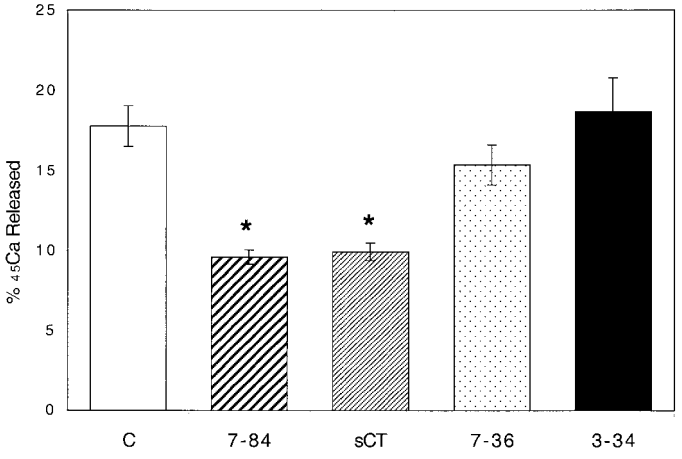

FIG. 1. Inhibition of basal bone resorption by hPTH-(7-84). Calvariae prelabeled with ^{45}Ca by maternal injection were isolated from neonatal mice as described in *Materials and Methods*. Bones were incubated individually, in treatment groups of four bones each, for 72 h after a single addition of vehicle alone (controls, C); hPTH-(7-84), hPTHrP-(7-36), or hPTH-(3-34) (all at 300 nM); or salmon calcitonin (sCT; 100 nM). Results are expressed as percentages of total ^{45}Ca released over 72 h. Values shown are the mean ± SEM of results from several (three to six) independent experiments. *, $P < 0.001$ *vs.* controls.

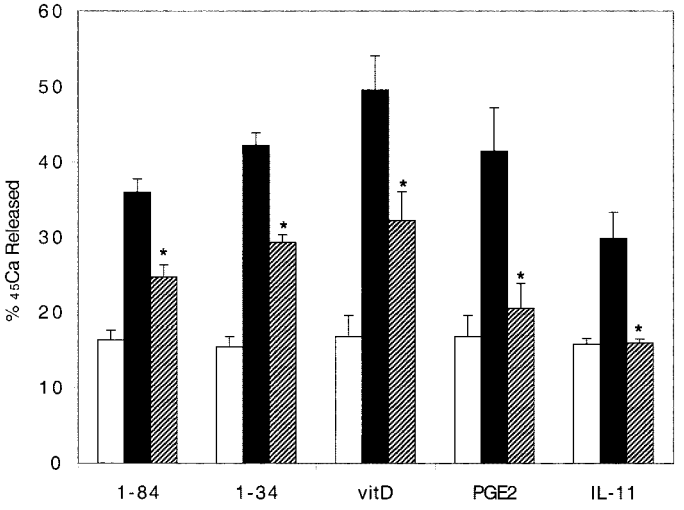

FIG. 2. Inhibition by hPTH-(7-84) of bone resorption induced by various osteotropic agents. Resorption assays were conducted as described in Fig. 1 for controls (□), osteotropic agents alone (■), or osteotropic agents in combination with 300 nM hPTH-(7-84) (▨). Osteotropic agents were employed at the following concentrations: hPTH-(1-84), 3 nM; hPTH-(1-34), 3 nM; VitD, 10 nM; PGE_2, 100 nM; and IL-11, 10 ng/ml. Results are expressed as the mean ± SEM of quadruplicate determinations. Each experiment was repeated three times. *, $P < 0.05$ for difference between osteotropic agent alone *vs.* osteotropic agent plus hPTH-(7-84).

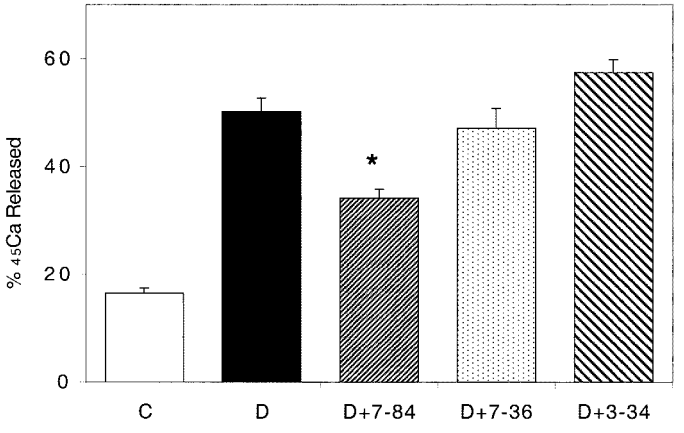

FIG. 3. Inhibition of VitD-induced bone resorption by hPTH-(7-84), but not by hPTHrP-(7-36) or hPTH-(3-34). Calvarial bones, incubated as described in Fig. 1, were treated with vehicle alone (C) or with 10 nM VitD, either alone (D) or together with 300 nM hPTH-(7-84) (D + 7-84), 1 μM hPTHrP-(7-36) (D + 7-36), or 1 μM hPTH-(3-34) (D + 3-34). Results are expressed as the mean ± SEM of quadruplicate determinations and are representative of three independent experiments. *, $P < 0.05$ between VitD alone and VitD plus PTH-(7-84).

resorptive response of the bones to subsequently added VitD (data not shown).

hPTH-(7-84) does not activate adenylyl cyclase or measurably inhibit the binding of a radiolabeled hPTH-(1-34) analog to PTH1Rs expressed on ROS 17/2.8 rat osteosarcoma cells, which also express CPTHRs (21, 27). To directly address the possibility that hPTH-(7-84) nevertheless might antagonize signaling by PTH1R agonists, either directly at the PTH1R or via activation of CPTHRs, ROS 17/2.8 cells were incubated with hPTH-(1-34) at a submaximal concentration (3 nM) in the absence or presence of excess hPTH-(7-84) (0.1–1 μM). As shown in Fig. 4, we observed no inhibition of the cAMP response to PTH-(1-34) by hPTH-(7-84) (0.1–1 μM), whereas both PTHrP-(7-36) and PTH-(3-34), when present at 1 μM, inhibited the response by 50%. In related experiments undiluted samples of conditioned medium from calvarial resorption assays (described above) to which 300 nM hPTH-(7-84), PTHrP-(7-36) or PTH-(3-34) had been added 72 h earlier also were tested for inhibition of hPTH-(1-34)-induced cAMP accumulation in ROS 17/2.8 cells. In none of these samples was inhibition of the cAMP response to hPTH-(1-34) observed (data not shown).

Inhibition of bone resorption by hPTH-(7-84) could result from decreased osteoclast formation, inhibition of the activity or survival of mature osteoclasts, or both. To determine whether hPTH-(7-84) impairs osteoclast formation, the activity of this fragment was studied in cultures of whole murine bone marrow. As shown in Fig. 5A, hPTH-(7-84) alone exerted no effect on the formation of TRAP+MNCs, although basal osteoclast formation in this system is low, and an inhibitory effect therefore might not be easily detectable. On the other hand, when osteoclast formation was stimulated by VitD (10 nM), hPTH-(7-84) (300 nM) caused a striking (70%) reduction in the formation of TRAP+MNCs relative to the effect of VitD alone [VitD, 153 ± 38 cells; VitD + hPTH-(7-84), 53 ± 14 cells]. In contrast,

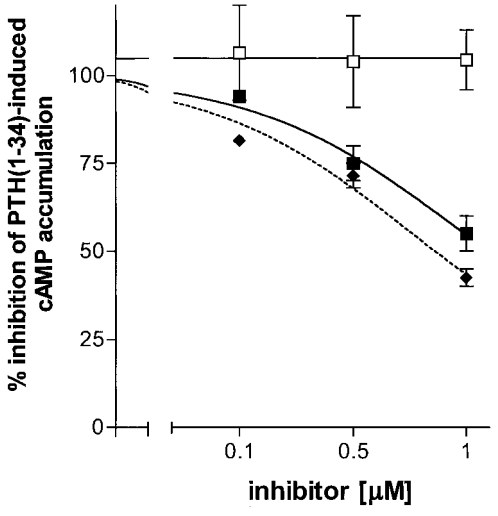

FIG. 4. Inhibition of cAMP accumulation in PTH-treated ROS 17/2.8 cells. Cells were stimulated with an approximately half-maximal concentration of PTH-(1-34) (3 nM) in the absence or presence of increasing concentrations of PTH-(7-84) (□, *solid line*), PTH-(3-34) (◆, *dashed line*), or PTHrP-(7-36) (■, *solid line*). Data are expressed as percentages of the cAMP response to 3 nM PTH-(1-34) alone and represent the results (mean ± SEM) of at least two independent experiments.

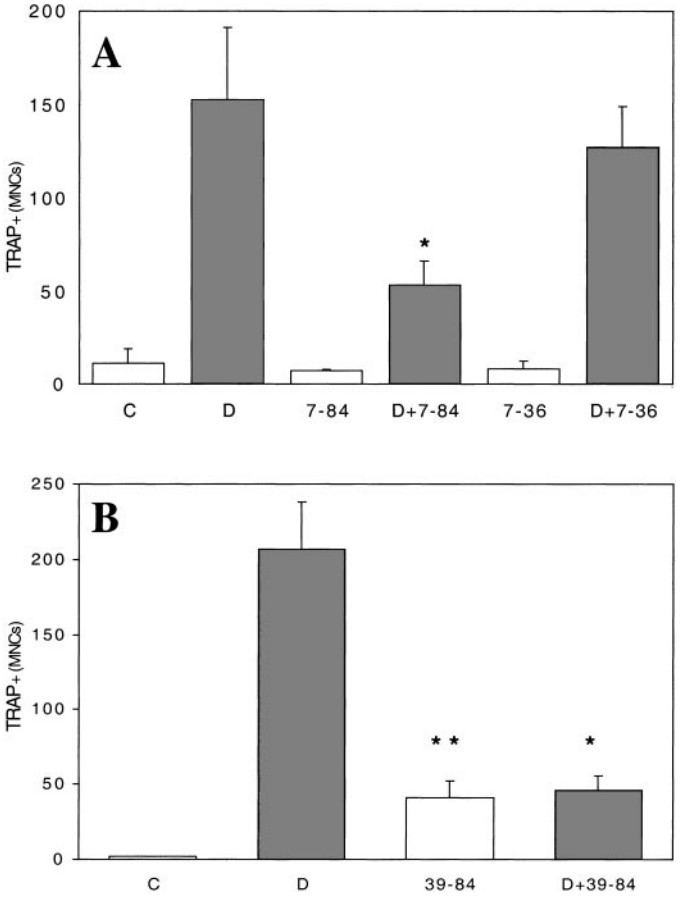

FIG. 5. Inhibition of osteoclast generation by hPTH-(7-84) and hPTH-(39-84). Whole bone marrow was isolated and cultured as described in *Materials and Methods*. Adherent and nonadherent cells were maintained in culture for 12 d, and the indicated treatments were added three times weekly, as described in *Materials and Methods*. At the end of the culture period, cells were fixed and stained for TRAP. A: C, Control; D, VitD (10 nM); 7-84, hPTH-(7-84) (300 nM); 7-36, hPTHrP-(7-36) (300 nM). B: C, Control; D, VitD (10 nM); 39-84, hPTH-(39-84) (3000 nM). Values (number of cells per well) are expressed as the mean ± SD for triplicate determinations (see *Materials and Methods*). Experiments were repeated twice. *, $P < 0.05$ *vs.* VitD alone; **, $P < 0.05$ *vs.* control.

PTHrP-(7-36) did not inhibit TRAP+MNC formation induced by VitD [VitD + PTHrP-(7-36), 127 ± 22 cells; Fig. 5A]. To determine whether shorter C-terminal PTH fragments could regulate osteoclast formation, we tested the effects of hPTH-(39-84), alone or in combination with VitD (10 nM). As shown in Fig. 5B, hPTH-(39-84) alone, at 3000 nM, slightly stimulated osteoclast formation (41 ± 11 cells), as reported previously for short CPTH fragments (24, 33). Like hPTH-(7-84), however, hPTH-(39-84) dramatically inhibited osteoclast formation promoted by VitD [VitD, 207 ± 31 cells; VitD + hPTH-(39-84), 46 ± 10 cells].

Discussion

Recent studies demonstrating that the extended CPTH fragment hPTH-(7-84) can inhibit the calcemic effects of PTH-(1-84) and PTH-(1-34) in thyroparathyroidectomized animals have suggested that CPTH peptides, normally present in blood and previously assumed to be biologically inert prod-

ucts of PTH metabolism, may be physiologically active (20, 21). As the hypocalcemic actions of hPTH-(7-84) described *in vivo* were associated with lowering of serum phosphate but were not accompanied by significant changes in urinary calcium or phosphate excretion (20, 21), a primary effect of this CPTH peptide on bone seems likely. The present *in vitro* studies were directed at clarifying whether hPTH-(7-84) might act directly on bone to inhibit the action of hPTH-(1-84) or hPTH-(1-34).

One possible mechanism for such an effect could involve direct antagonism by hPTH-(7-84) to prevent binding of hPTH-(1-34) or hPTH-(1-84) to PTH1Rs expressed on osteoblasts or marrow stromal cells. Because the antagonism *in vivo* is observed at much lower doses of hPTH-(7-84), relative to intact PTH, than that predicted to be necessary for direct antagonism at the PTH1R, however, we also considered the alternative possibility that hPTH-(7-84) may exert unique PTH1R-independent antiresorptive effects by activating CPTHRs expressed in bone cells. Our results are fully consistent with this latter hypothesis. Thus, we observed concentration-dependent inhibition of bone resorption in *ex vivo* calvarial organ cultures that was not mimicked by shorter, N-truncated PTH fragments that 1) are more effective PTH1R antagonists than is hPTH-(7-84) and 2) do not bind detectably to CPTHRs expressed on bone cells (28). Similar results were obtained in studies of osteoclastogenesis using whole bone marrow cultures, which further suggests that CPTHRs may be involved in the regulation of osteoclast formation. Because the number of mononuclear TRAP-positive cells formed in the marrow cultures also was reduced by hPTH-(7-84), the predominant action in osteoclastogenesis may be to inhibit formation of osteoclast precursors. The rapidity (1–2 h) of the hPTH-(7-84) effect observed *in vivo*, however, suggests that interference with the activity of mature osteoclasts also may be involved.

The antiresorptive effect of hPTH-(7-84) observed in the calvarial assay system contrasted sharply with the inability of hPTH-(3-34) or PTHrP-(7-36), introduced at similar concentrations, to inhibit resorption. Because both of these shorter, N-truncated peptides are effective *in vitro* PTH1R antagonists, whereas hPTH-(7-84) is not (as shown in Fig. 4), these results argue strongly against a mechanism involving direct antagonism by hPTH-(7-84) at the PTH1R of either endogenous PTHrP present within the cultured bones or exogenously added PTH. Moreover, the antiresorptive effect of hPTH-(7-84) *in vitro* was not restricted to resorption induced by added PTH, but was encountered in both control cultures and cultures treated with a variety of unrelated bone-resorbing agonists, including VitD, PGE_2 and IL-11. These findings point to a more generalized antiresorptive mechanism by which PTH-(7-84), presumably acting via CPTHRs, may limit the formation and, possibly, the activity of mature osteoclasts. This could reflect interference with the up-regulation of RANKL or macrophage colony-stimulating factor, the down-regulation of OPG, or both, that normally are triggered in marrow stromal cells and osteoblasts by these diverse resorbing agents (35). In this regard, we observed expression of CPTHRs by PTH1R-null osteoblasts and osteocytes (28) and by clonal marrow stromal cells that are capable of supporting PTH- or vitamin D-dependent osteoclast formation from hemopoietic progenitors *in vitro* (our unpublished observations). We also cannot yet exclude that the inhibition of resorption was mediated partly by a proapoptotic effect of hPTH-(7-84) on bone cells via activation of the CPTHR, as we previously reported in osteocytic cells (28). Moreover hPTH-(7-84) could act directly on mature osteoclasts, their hemopoietic precursors, or both to dampen cellular responsiveness to activation of RANK or c-*Fms* by their respective stromal cell or osteoblast-derived ligands. Indeed, evidence that shorter CPTH fragments alone can modestly induce osteoclast formation, as seen in the present study with hPTH-(39-84) and reported previously (24, 33), in contrast to the inhibitory effects of the same fragment upon osteoclast formation induced by vitamin D, points to a complexity in CPTHR action that is not readily explained at present, but that could involve disparate effects on distinct cell types involved in osteoclast formation. Direct analysis of CPTHR expression in such cells would be needed to address this possibility.

One prediction of our results might be that PTH-(1-84), which binds to CPTHRs with affinity comparable to that of PTH-(7-84) (21), should elicit less bone resorption than PTH-(1-34), which does not interact effectively with CPTHRs (28). Although few direct *in vitro* comparisons have been performed (24, 36), the available data do not indicate substantial or consistent differences in resorptive responses to these two peptides. Indeed, we observed in the calvarial resorption assay that the intact hormone reproducibly induced less ^{45}Ca release than did PTH-(1-34) at equimolar concentrations, although this difference was never statistically significant. This could indicate that when PTH1Rs and CPTHRs are exposed simultaneously to equimolar concentrations of a common ligand, the PTH1R-mediated resorptive response strongly predominates. Alternatively, it is possible that despite comparable binding affinity, intact PTH cannot activate CPTHRs as effectively as N-truncated peptides (by analogy with the disparate activation of PTH1Rs observed with PTH-(1-34) *vs.* PTH-(3-34)). On the other hand, because CPTH fragments normally circulate in plasma at concentrations at least 5- to 10-fold higher than those of intact PTH, a requirement for higher molar concentrations of CPTH ligands to activate CPTHRs might be expected. This concept is consistent with our finding that a 10- to 100-fold molar excess of CPTH ligand is needed to elicit functional antagonism of PTH1R-mediated resorption *in vitro*. The observation that hPTH-(7-84) could antagonize the calcemic response to hPTH-(1-84) at equimolar doses *in vivo* might be related to differential bioavailability or metabolism of the two peptides after their ip or iv administration (20, 21).

Secretion of CPTH fragments by the parathyroid glands is positively regulated by blood calcium (37). Thus, one possible physiological role of the antiresorptive action of N-truncated PTH fragments *in vivo* could be to modulate the extent of bone resorption induced by intact PTH in a manner responsive to the extracellular calcium concentration. Such a mechanism, for example, might allow for maximal release of calcium from bone only during severe hypocalcemia to supplement the ongoing renal and (indirect) intestinal actions of PTH. It is important to note that the chemical identities of all circulating CPTH fragments have not yet been completely defined. In particular, the existence in blood of PTH-(7-84) *per se* has not been directly demonstrated. On the other hand, the

recent immunochemical characterization of nonintact PTH peptides, which are especially abundant in renal failure, is consistent with the presence of extended CPTH fragments longer than those previously inferred from analyses of secreted or peripherally generated cleavage products, the N-termini of which ranged between positions 24 and 43 of the intact PTH sequence (19, 38). Thus, the possibility that PTH fragments similar or identical to PTH-(7-84) may be present in blood, especially in renal failure, at concentrations high enough to activate CPTHRs and thereby exert direct effects on bone resorption must be considered.

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